CIITA-Induced MHC Class II Expression in Mammary Adenocarcinoma Leads to a Th1 Polarization of the Tumor Microenvironment, Tumor Rejection, and Specific Antitumor Memory

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Abstract

Purpose: We have shown previously that the MHC class II–negative murine TS/A adenocarcinoma is rejected in vivo if induced to express MHC class II molecules by transfection of the MHC class II transactivator CIITA. In this study, we explored the immunologic basis of tumor rejection and the correlation between histopathology of tumor tissue and immune rejection.

Experimental Design: Stable TS/A-CIITA transfectants were generated and injected into mice. In vivo cell depletion, immunohistochemistry of tumor tissues, and immune functional assays were done to assess the cellular and immunologic basis of rejection.

Results: Ninety-two percent of mice injected with TS/A-CIITA rejected the tumor and were completely resistant to challenge with parental TS/A. Only CD4+ and CD8+ cells were required for rejection. The tumor microenvironment in TS/A-CIITA-injected mice changed dramatically when compared with the TS/A parental-injected mice. Rapid infiltration with CD4+ T cells followed by dendritic cells, CD8+ T cells, and granulocytes was observed. Importantly, TS/A-CIITA cells could act as antigen-presenting cells because they process and present nominal antigens to CD4+ T cells. Tumor-specific CD4+ T cells of TS/A-CIITA-injected mice had the functional characteristics of Th1 cells and produced IFN-γ and this was relevant for generation and maintenance of protective antitumor response, because IFN-γ knockout mice were no longer rejecting TS/A-CIITA tumor cells.

Conclusion: CIITA-dependent MHC class II expression confers to TS/A tumor cells the capacity to act as a protective vaccine against the tumor by triggering tumor antigen presentation to T helper cells, antitumor polarization of cellular and soluble components of the tumor microenvironment, and establishment of antitumor immune memory.

It is widely accepted that the immune system can recognize and respond against neoplastic cells (1). However, the clinical history of a cancer patient often shows the failure of the immune system to eliminate the tumor. Events connected with the histologic origin of the tumor, the newly acquired phenotype of tumor cells and the functional phenotype of the tumor stroma, can result in a polarized microenvironment more favorable to tumor growth than tumor rejection (2–6).

Several attempts have been made to modify the antitumor immune response and the tumor environment either by tumor-specific MHC class I–restricted peptide vaccination for rescuing CTL activity or by increasing the availability of soluble mediators, including cytokines and chemokines (7). The CTL responses were generally weak and unable to control tumor growth in most patients when using CTL-defined antigens as vaccines in clinical trials (8). It is now clear that this is mostly due to poor tumor-specific, MHC class II–restricted CD4+ T cell help generated in tumor-bearing patients (9). In fact, T helper (Th) cells are required for optimal induction of both humoral and cellular effector mechanisms (10, 11) and Th cells...
expressing CD40L are necessary for inducing CTL cross-priming by dendritic cells (12). Most attempts to produce relevant Th-derived cytokines and other soluble mediators in the tumor microenvironment have been mainly carried out by genetic transfer of relevant cDNAs into tumor cells. A variety of cytokines and chemokines may lead to tumor regression both by direct antiangiogenic effects as in the case of IFN-γ (13) and by activation of antitumor immunity. Nevertheless, clinical application is seriously hampered by the difficulty in controlling, in terms of both amount and duration, the efficacy and the possible adverse effects generated by the release of the biological mediators (6).

Few attempts have been focused on modifying the initial phases of the immune recognition centered on tumor antigen presentation to Th cells (14). The use of professional antigen-presenting cells (APC), such as dendritic cells, pulsed with tumor antigen peptides in vitro and reinfected in vivo has been limited to those cases where we know the relevant tumor antigen generating an efficient immune response in vivo and requires high dendritic cell numbers to be efficacious (15). An alternative possibility would be to render the tumor cells themselves surrogate APCs by providing them with stable expression of the MHC class II molecules, which bind antigenic peptides and present them to CD4+ T cells (16). We recently described that transfection of murine TS/A mammary adenocarcinoma cells (TS/A-pc) with the AIR-1 locus-encoded MHC class II transactivator, CIITA (17–19) rendered them MHC class II I-A and I-E positive. Fifty percent of the tumors were rejected in vivo. The rejection involved the participation of CD4+ T cells, necessary for the priming phase, and CD8+ antigen-specific CTLs as effectors cells (20). Here, we have investigated in detail the in vivo correlates of tumor rejection. We show that stable expression of CIITA in tumor cells results in complete rejection in essentially all injected animals and establishment of a long-lasting antitumor memory against TS/A-pc. Importantly, we show that TS/A-CIITA cells can process and present nominal antigens to antigen-specific CD4+ T cells, and once injected in vivo, they induce a rapid and dramatic change of the tumor microenvironment resulting in a rapid infiltration of the tumor with CD4+ T cells and dendritic cells followed by CD8+ T cells and granulocytes. Finally, we defined early tumor-specific CD4+ T cells secreting IFN-γ as the key Th1 regulators leading to tumor rejection and specific antitumor memory.

**Materials and Methods**

**Cell lines, CIITA transfection, and cell surface phenotyping.** TS/A mammary adenocarcinoma (kindly provided by P.L. Lollini, University of Bologna, Bologna, Italy; ref. 21), C26 colon carcinoma (22), and F1F fibrosarcoma (23) murine cell lines were cultured in DMEM supplemented with L-glutamine, HEPES, and 10% fetal bovine serum in a 5% CO2 atmosphere at 37°C. Transfection of TS/A with CIITA, isolation of stable CIITA-expressing clones, and MHC cell surface phenotype of transfecteds have been described previously (20, 24).

**Animal model, experimental conditions, and depletion procedures.** Five-week-old female BALB/c (H-2k) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Animals were injected s.c. with 1×106 either parental (TS/A-pc), empty vector-transfected (TS/A-hyg), or CIITA-transfected (TS/A-CIITA) tumor cells. Rechallenge with the same tumorigenic dose of TS/A-pc in TS/A-CIITA-rejecting animals was carried out s.c. in the opposite flank. Tumor size was measured using a caliper at weekly intervals and was expressed as a multiple of the wider and smaller tumor diameters. For in vivo depletion, spleen cells were treated with either anti-CD4 (GK1.5) or anti-CD8 (2.43) rat monoclonal antibodies (mAb) and complement (Cederlane, Hornby, Ontario, Canada) as described (20). Isotype-matched mAbs of unrelated specificity were used as controls. Cell subset in vivo depletions in TS/A-CIITA-inoculated mice were done as described previously (20) by i.p. injections of either 300 μg anti-CD4 (GK1.5; American Type Culture Collection, Rockville, MD) or 300 μg anti-CD8 (2.43; American Type Culture Collection) rat mAbs, anti-asialo-GM1 [anti–natural killer (NK) rabbit serum; 200 μL/mouse of 1:10 diluted stock solution; Wako], anti-Gr-1 rat mAb (clone RB6-8C5; 200 μL of a 1:50 diluted ascites; kindly provided by Dr. R. Coffman, DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, CA), or 300 μg anti-B rat mAb (clone RA3-3A1/6.1; American Type Culture Collection). Control animals received normal rabbit serum or an irrelevant rat mAb as described (20).

Depletion efficiency for each cellular subset for either in vivo or in vitro treatment was always >95% as assessed by immunofluorescence and fluorescence-activated cell sorting analysis (Becton Dickinson, Milan, Italy) on splenocytes of euthanized mice.

**Immunohistochemical analyses.** Groups of three mice each were euthanized at 5, 7, and 10 days after s.c. tumor inoculation of either TS/A or TS/A-CIITA. Cryostat sections (6 μm thick) were air-dried and fixed in cold acetone for 10 minutes. Immunostaining was done using a streptavidin-biotin-alkaline phosphatase complex staining kit (Bio-Spa Division, Milan, Italy) and naphthol-AS-MX-phosphate and Fast Red TR (Sigma, St. Louis, MO) to visualize binding sites. The mAbs used were anti-CD4 (GK1.5), anti-CD8 (2.43), anti–dendritic cells (DEC-205; clone A01D-145; ImmunoKontact, Lugano, Switzerland), anti-polymorphonucleate Ly-6G (Gr-1; clone RB6-8C5), anti-macrophage (clone MOMA-1; ImmunoKontact), anti-B (clone RA3-3A1/6.1), and anti-NK (anti-asialo-GM1). The sections were incubated with the primary antibody overnight at 4°C. The red reaction product was obtained using a mixture of 2 mg naphthol-AS-MX-phosphate dissolved in 200 μL N,N-dimethylformamide (Sigma) and diluted in 9.8 mL of 0.1 mol/L Tris-HCl (pH 8.2) and 1 mL 1% levamisole (Sigma). Immediately before use, 10 mg Fast Red TR salt was added. Gill’s hematoxylin was used as a counterstain and the sections were mounted in glycerol (DAKO, Carpenteria, CA). Quantiative studies of stained sections were done independently by three pathologists in a blind fashion. Cell counts were obtained in 8 to 12 randomly chosen fields under a Leica Wetzlar light microscope (Solms, Germany) at ×400 magnification, 0.180 mm2/field. Mann-Whitney U-test was used to evaluate whether there was a statistically significant difference between TS/A-pc and TS/A-CIITA cellular infiltrate. Data analyses were considered highly significant when P < 0.005.

**Antigen presentation assay.** TS/A-CIITA 32.10.7 (TSA-CIITA), TS/A-pc, or BALB/c adherent peritoneal macrophages were used as APCs. The cells were seeded in triplicates of a 96-well plate at the concentrations indicated and incubated for 4 hours with either chicken ovalbumin or the ovalbumin peptide 323–339 (kindly donated by Dr. G. P. Corradin, University of Lausanne, Lausanne, Switzerland). A fixed number of I-Ak-restricted, ovalbumin 323–339-specific DO11.10 T-cell hybridoma cells (5×104 per well; ref. 26), used as responder, were then added for 24 hours. The optimal concentrations of ovalbumin protein and ovalbumin peptide used in the assay were established after titration and were 50 and 10 μg/mL, respectively. To block antigen processing (27), parallel cultures of TS/A-CIITA cells were preincubated with chloroquine at 10 μmol/L final concentration for 2 hours at 37°C, and chloroquine was washed away before addition of ovalbumin protein/ovalbumin peptide.

At the end of the incubation, 50 μL culture supernatant derived from the various cultures were tested for interleukin (IL)-2 production on 104 CTLL-2 cells per well. Briefly, CTLL-2 cells were incubated with the various supernatants for 18 hours, 1 μCi [3H]thymidine was added, and
incubation was allowed for additional 6 hours. Cultures were harvested using a Harvester 96 (Tomtec, Inc., Hamden, CT) and thymidine incorporation (counts/min) was measured using a 1450 MicroBeta counter (Wallac, Turku, Finland).

**Enzyme-linked immunospot assays.** Enzyme-linked immunospot assay was done using ex vivo splenocytes from either naive or treated mice at 2 weeks postinoculation. Multiscreen-IP plates (Millipore, Bedford, MA) were coated overnight with 10 μg/mL of either anti-IFN-γ or anti-IL-4 mAbs in PBS (Endogen, Woburn, MA). Plates were then washed with RPMI 1640 and blocked for 3 hours with PBS-2% bovine serum albumin. Splenocytes were resuspended in complete RPMI 1640 and then seeded at 2-fold serial dilution starting from 3 × 10⁵ cells per well in triplicate in the presence or absence of irradiated (20,000 rad) TS/A-pc, TS/A-CIITA, C26, or F1F cells at 10:1 effector/stimulator cell ratio. After 40 hours of incubation, plates were washed with PBS-0.05% Tween 20 and incubated with 1 μg/mL biotinylated secondary mAb to IFN-γ or IL-4 mAbs (Endogen) in PBS-1% bovine serum albumin for 3 hours at room temperature. Then, horseradish peroxidase–conjugated streptavidin (1:5,000) was added for 2 hours at room temperature. After washing, the plates were stained with AEC staining kit (Sigma) and spots were counted using a stereomicroscope. A >2-fold increase of number of spots over the control was considered as a positive response.

**Results**

**Stability of CIITA expression dramatically increases tumor rejection in vivo and protection from challenge.** From a previously described TS/A-CIITA transfectant (32.10; ref. 20), we isolated several sublines showing strong and stable expression of MHC class II I-A and I-E cell surface molecules over months in culture (Table 1). All sublines and parental 32.10 cells displayed similar growth kinetics in vitro, and apart from the MHC class II cell surface phenotype and invariant chain expression (20), they did not show any other significant phenotypic variation with respect to the untransfected (TS/A-pc) or vector-transfected (TS/A-Hygro) cells. All TS/A-CIITA 32.10-derived cell lines were rejected in vivo by a higher percentage of injected mice compared with the original transfectant and this correlated with their higher and stable expression of MHC class II cell surface molecules. Moreover, rejecting mice were resistant to challenge with the parental TS/A-pc tumor. One subline, 32.10.7, was rejected in 92% of the injected animals (Table 1) and was selected for further study.

<table>
<thead>
<tr>
<th>Line</th>
<th>MHC class II</th>
<th>MHC class I</th>
<th>No. tumor-injected mice</th>
<th>Mice rejecting the tumor (%)</th>
<th>Rejecting mice resistant to challenge with parental TS/A</th>
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</thead>
<tbody>
<tr>
<td>TS/A-CIITA 32.10</td>
<td>238*</td>
<td>293*</td>
<td>216*</td>
<td>45</td>
<td>23 (51)</td>
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<tr>
<td>TS/A-CIITA 32.10.7</td>
<td>1,516</td>
<td>1,658</td>
<td>317</td>
<td>25</td>
<td>23 (92)</td>
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<tr>
<td>TS/A-CIITA 32.10.9</td>
<td>1,020</td>
<td>1,312</td>
<td>280</td>
<td>8</td>
<td>6 (75)</td>
</tr>
<tr>
<td>TS/A-CIITA 32.10.10</td>
<td>527</td>
<td>582</td>
<td>322</td>
<td>8</td>
<td>6 (75)</td>
</tr>
<tr>
<td>TS/A-CIITA 32.10.13</td>
<td>308</td>
<td>367</td>
<td>267</td>
<td>8</td>
<td>5 (62)</td>
</tr>
<tr>
<td>TS/A-pc</td>
<td>12</td>
<td>13</td>
<td>208</td>
<td>15</td>
<td>0 (0)</td>
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</tbody>
</table>

**Table 1. Tumor rejection correlates with the amount of CIITA-mediated MHC class II cell surface molecules**

**NOTE:** TS/A-CIITA-rejecting mice are uniformly resistant to challenge with TS/A parental cells.

*Mean fluorescence as assessed by specific mAb and cytofluorometric analysis.

Fraction of mice resistant to challenge with respect to the total number of mice challenged.

Superimposable results were obtained with TS/A-hygro cells.

Rejection of TS/A-CIITA correlates with the presence of CD4⁺ and CD8⁺ T cells and not with the presence of B cells, NK cells, and polymorphonucleates. Our initial studies indicated that the CD4⁺ T cells were necessary for priming and CD8⁺ T cells were required as effectors in the antitumor immune response against the CIITA-transfected TS/A tumor cells (20). However,
the possible contribution of other cell subsets was not investigated previously. We assessed the role of B cells, NK cells, polymorphonucleates, and again CD4⁺ and CD8⁺ T cells in tumor rejection by depriving naive animals of each of these cell subsets by injecting specific antibodies before 32.10.7 tumor cell injection. Removal of either B cells, NK cells, or polymorphonucleates did not affect the capacity of mice to reject the TS/A-CIITA tumor cells, as 88% of either

Fig. 2. Comparative immunohistochemical analysis of tumor tissues of animals injected with TS/A-pc or TS/A-CIITA 32.10.7 (TS/A-CIITA) cells. Cryostat sections of tumor tissues at days 5, 7, and 10 after cell injection were analyzed. Sections were stained with antibodies specific for CD4 (A), CD8 (B), dendritic cells (C), macrophages (D), or polymorphonucleates (E). Positive cells appear with red-purple staining over the counterstaining with Gill's hematoxylin. Diffuse areas of necrosis (nec) are visible in TS/A-CIITA tumors from day 7. Original magnification, ×400.
B-cell-deprived or polymorphonucleate-deprived and 100% of NK-deprived animals were still rejecting the 32.10.7 cells (Fig. 1A). Conversely, in vivo removal of either CD4+ or CD8+ T cells abrogated the tumor rejection capacity (Fig. 1B), confirming that the major populations involved in tumor rejection are CD4+ and CD8+ T cells.

Tumor tissue correlates of rejection. To establish the in vivo histopathologic correlates of rejection, TS/A-pc and TS/A-CIITA tumor tissues were analyzed by immunohistochemistry over time (Fig. 2) and the cellular infiltrate was quantified (Table 2) as described in Materials and Methods. Importantly, extensive areas of necrosis (Fig. 2, nec) were present as early as day 7 only in TS/A-CIITA tumors (bottom). CD4+ cells (Fig. 2A) were significantly detected at day 5 in TS/A-CIITA (d, arrow points to one of many) but not in TS/A-pc tumors (a; Table 2; P < 0.005). CD4+ T cells rapidly infiltrated the TS/A-CIITA tumor at day 7, and at day 10, they were present in a significant high number (Table 2) and in both viable and necrotic tumor stroma (f). In contrast, only rare CD4+ cells could be detected in TS/A-pc tumors and mostly at day 10 (c, arrow).

A similar, although not overlapping, picture was observed when tumor tissues were stained for CD8 antigen (Fig. 2B). In TS/A-CIITA tumors CD8+ cells, firstly detected on day 7 (e, arrow points to one of many), massively accumulated at day 10 around the necrotic area and intermingled with tumor cells showing clear signs of stress (f). Instead, tumors derived from TS/A-pc did not present a CD8+ T-cell infiltration even at the latest time point.

As observed for CD8+ T cells, in TS/A-CIITA tumor tissues, dendritic cells were almost undetectable at day 5 but were clearly present on day 7 (Fig. 2C, e, arrow points to one of many; Table 2). Although many dendritic cells were detected at this stage as interdispersed elements in the necrotic area, the vast majority of them were localized in the tumor area surrounding the necrotic tissue. At day 10, a massive infiltration by dendritic cells of this area was apparent (f). In contrast, TS/A-pc-derived tumor tissues did not show significant presence of dendritic cells at any of the three time points analyzed (Table 2).

No evident difference in macrophage numbers was observed between TS/A-CIITA and TS/A-pc tumor tissues (Fig. 2D). Both
Infiltration on day 7 (tissue there was a drastic increase in polymorphonucleate
around and within the necrotic areas (tissue was mostly necrotic, and macrophages were still visible
day 7. Interestingly, at day 10, when the TS/A-CIITA tumor
arrow points to one of many). The infiltration was increased on
infiltration remained quantitatively similar at days 5 and 7 (whereas in TS/A-pc tumor tissue the polymorphonucleate
macrophages was drastically reduced compared with day 7.

Very few B cells and NK cells were present in either TSA-pc or
TS/A-CIITA tumors and their number did not change over time
(data not shown).

Polymorphonucleates were present in both tumor tissues
(Fig. 2E; Table 2). They were interdispersed within the tumor
(Fig. 2E, arrows point to one of many). However, whereas in TS/A-pc tumor tissue the polymorphonucleate
infiltrate remained quantitatively similar at days 5 and 7 (a and b) but was reduced at day 10 (c), in TS/A-CIITA tumor
tissue there was a drastic increase in polymorphonucleate infiltration on day 7 (c; P < 0.005), mostly localized around
the tumor. At day 10, all TS/A-CIITA tumor tissue was still
infiltrated by polymorphonucleates, although most of the
polymorphonucleates were dead and included in the vast
necrotic area characterizing the tumor tissue at this stage (f).

TS/A-CIITA cells process and present nominal antigens to MHC
class II–restricted Th cells. To assess whether de novo expression
of MHC class II genes on CIITA-transfected cells had
functional relevance, we tested their capacity to present the
ovalbumin antigen to the MHC class II I-A\(^d\)-restricted,
ovalbumin-specific T-cell hybridoma line DO11.10. The
CIITA-transfected TS/A cells pulsed with the helper ovalbumin
peptide 323-339 stimulated the specific T-cell hybridoma,
indicating antigen-presenting competence (Fig. 3A). Very
importantly, DO11.10 T cells were also stimulated when
TS/A-CIITA cells were pulsed with the whole ovalbumin but
not when the ovalbumin pulsing was done after treatment
with chloroquine, an agent that blocks acidification and
proteolytic degradation in the endosomal compartment
(Fig. 3B). As expected, chloroquine treatment did not
affect the APC capacity of TS/A-CIITA cells pulsed with the
ovalbumin peptide 323-339 (Fig. 3A). These results show
antigen-processing function in the CIITA-transfected tumor
cells.

Functional phenotype of tumor-specific T cells in TS/A-CIITA-
rejecting mice at time of tumor resolution. We then assessed
whether the dramatic change in the tumor microenvironment
and the potent immune rejection response generated against
the TS/A-CIITA tumor correlated with a functional polarization
of the Th cells. When spleen cells of tumor-rejecting mice
(TS/A-CIITA spleen cells in Fig. 4) at 2 weeks after tumor
inoculation, early after tumor resolution, were stimulated

<p>| Table 2. Immunohistochemical analysis of infiltrating cells in the TS/A-pc and TS/A-CIITA tumor microenvironment at different time points from s.c. tumor cell injection in BALB/mice |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Infiltrating cells(^*)</th>
<th>Day 5</th>
<th>Day 7</th>
<th>Day 10</th>
<th>Day 5</th>
<th>Day 7</th>
<th>Day 10</th>
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<tr>
<td>CD4</td>
<td>1.2 ± 1.4</td>
<td>9.9 ± 3.6(^1)</td>
<td>1.8 ± 2.3</td>
<td>32.5 ± 12.1(^1)</td>
<td>4.7 ± 3.7</td>
<td>42.0 ± 17.7(^1)</td>
</tr>
<tr>
<td>CD8</td>
<td>1.0 ± 1.5</td>
<td>1.0 ± 1.0</td>
<td>0.8 ± 0.7</td>
<td>4.9 ± 1.6(^1)</td>
<td>1.7 ± 1.4</td>
<td>40.8 ± 12.4(^1)</td>
</tr>
<tr>
<td>Dendritic cells</td>
<td>0.8 ± 0.9</td>
<td>1.5 ± 1.1</td>
<td>2.1 ± 2.3</td>
<td>38.6 ± 16.5(^1)</td>
<td>1.0 ± 0.9</td>
<td>67.6 ± 16.3(^1)</td>
</tr>
<tr>
<td>Macrophages</td>
<td>7.8 ± 3.1</td>
<td>8.7 ± 6.2</td>
<td>22.5 ± 9.1</td>
<td>17.8 ± 4.4</td>
<td>8.5 ± 5.1</td>
<td>15.5 ± 8.0(^1)</td>
</tr>
<tr>
<td>Polymorphonucleates</td>
<td>21.8 ± 9.3</td>
<td>20.9 ± 9.7</td>
<td>20.5 ± 4.1</td>
<td>40.1 ± 11.3(^1)</td>
<td>13.7 ± 6.6</td>
<td>25.1 ± 13.7(^1)</td>
</tr>
</tbody>
</table>

*Cell counts at ×400 magnification in a 0.180-mm\(^2\) field. At least 8 to 12 randomly chosen fields per sample were evaluated.
\(^1\) P < 0.005, significantly different from corresponding values of TS/A-pc tumor grown in BALB/c mice at the same time point.
in vivo with TS/A-CIITA (Fig. 4, black columns), a high frequency of lymphocytes producing IFN-γ (Fig. 4A, left) and absence of lymphocytes producing IL-4 (Fig. 4B, left) were observed. The above spleen cells were stimulated to produce IFN-γ, although at reduced frequency, also by C26 colon carcinoma cells (Fig. 4A, left, hatched column), which share tumor-associated antigens with TS/A-pc tumor cells (28), but not by the antigenically unrelated F1F fibrosarcoma cells (Fig. 4A, left, dotted column). On the contrary, no IFN-γ-specific response was observed against any of the challenging tumor cells when spleen cells from TS/A-pc-injected mice were tested at 2 weeks after tumor inoculation (Fig. 4A, right, TS/A-pc spleen cells). Of relevance, in vitro depletion of either CD4+ (CD4 depl.) or CD8+ (CD8 depl.) T cells from spleens of TS/A-CIITA injected mice (A, inset) Results are number of spot-forming cells (SFC) per 10^6 total spleen cells used in the various conditions (Y axis). Columns, mean of triplicate cultures of three separate experiments; bars, SD.

**Discussion**

Our results clearly show that in a murine model of mammary adenocarcinoma cells the coordinate expression of endogenous MHC class II molecules induced by stable transfection with the MHC class II transactivator CIITA results in complete rejection of the tumor in virtually all injected mice. Apparently, CD4+ the triggering of tumor-specific CD4+ T cells displaying a Th1 phenotype in contrast to a skewed Th2 phenotype response observed in mice injected with TS/A-pc.

**GKO mice do not reject TS/A-CIITA tumor cells.** To further substantiate the importance of IFN-γ-secreting cells in mice rejecting TS/A-CIITA tumors and thus of the Th1 phenotype, GKO BALB/c mice were injected with TS/A-CIITA cells and monitored for tumor growth. Unlike their normal counterparts, GKO mice were not able to reject TS/A-CIITA tumor cells at all (Fig. 5A). Interestingly, both the appearance and the growth kinetics in vivo of the TS/A-CIITA tumors were virtually the same as those observed in immunocompetent BALB/c mice injected with TS/A-pc (Fig. 5B). Thus, IFN-γ not only identifies the phenotype of CD4+ T cells playing a major role in immunity against TS/A-CIITA tumor cells but also is required for the acquisition and/or maintenance of the antitumor activity.
CD4+ T cells responding to TS/A-CIITA but not to TSA-pc cells. Phases of TS/A-CIITA rejection show a very high frequency of absence of dendritic cells and CD8+ T cells. In contrast, CD4+ cell necrosis possibly initiated by the CD8+ CTL. The fact accompanied by the appearance of extensive areas of tumor infiltrated the tumors originated by TS/A-CIITA cells. This was CD8+ T cells and dendritic cells, along with the capacity of CD4+ T cells, supports the hypothesis that much of the tumor-presented without processing. (Directly bound by cell surface MHC class II molecules and nants present in the ovalbumin protein preparation were chloroquine, ruling out the possibility that peptide contami- cians presented in the ovalbumin protein preparation were directly bound by cell surface MHC class II molecules and presented without processing. (b) Spleen cells of mice in early phases of TS/A-CIITA rejection show a very high frequency of CD4+ T cells responding to TS/A-CIITA but not to TSA-pc cells.

In considering the role of TS/A-CIITA as surrogate APCs, it should be emphasized that TS/A tumor cells have an intrinsic capacity to release tumor-derived exosomes (33); in TS/A-CIITA, these exosomes would be rich in MHC class II tumor-associated antigen peptide complexes, and on phagocytosis by dendritic cells, these structures could be used by dendritic cells to complement their otherwise insufficient APC function for both priming and maintaining Th cell stimulation. Furthermore, it should be underlined that TS/A tumor cells are highly metastatic (21); thus, they have a natural propensity to migrate through lymphatics to draining lymph nodes. If TS/A-CIITA cells follow the same rule, after migration to lymph nodes, they may act as APCs or favor professional APC function as described above.

The comparative study of the tumor microenvironment in mice injected with TS/A-pc or TS/A-CIITA tumor cells provides critical insight on the mechanisms triggered by TS/A-CIITA tumor cells and their possible role as surrogate APCs. Tumors derived from TS/A-pc presented a scarce infiltrate, represented by macrophages and neutrophils, very few CD4+ T cells, and absence of dendritic cells and CD8+ T cells. In contrast, CD4+ T cells followed by dendritic cells and CD8+ T cells rapidly infiltrated the tumors originated by TS/A-CIITA cells. This was accompanied by the appearance of extensive areas of tumor cell necrosis possibly initiated by the CD8+ CTL. The fact that CD4+ T cells colonized TS/A-CIITA tumor tissue before CD8+ T cells and dendritic cells, along with the capacity of TS/A-CIITA cells to process and present antigenic peptides to CD4+ T cells, supports the hypothesis that much of the tumor-specific CD4+ T-cell triggering and/or restimulation takes place in the tumor tissue itself and is directly mediated by MHC class II tumor cells as has been suggested by previous studies (29, 30, 34).

Macrophages infiltration was essentially similar to that observed in TS/A parental tumors. In contrast, polymorphonucleate infiltration in TS/A-CIITA tumor tissue became remarkably high on day 7 and continued to day 10. Presentation of tumor antigens, either directly by the TS/A-CIITA or indirectly by dendritic cells having phagocytosed tumor vesicles or necrotic tumor cells, may activate CD4+ T cells to release a series of cytokines, chemokines, and other relevant mediators, which may further attract and activate polymorphonucleates as has been shown for TS/A-pc cells engineered with diverse cytokines and chemokines (35–38). Although the presence of polymorphonucleates was not required for inducing the rejection phenomenon, intratumor polymorphonucleates may participate in tumor cell killing as dramatically shown by our immunohistochemical studies. The excess necrotic material produced in the tumor mass may play a critical role in fueling professional APCs, such as dendritic cells, with large amounts of tumor-associated antigens. Dendritic cells could then maintain stimulation of specific antitumor CD4+ Th cells and CD8+ effector CTLs leading to the establishment of a critical reservoir of memory effector cells responsible for the accelerated rejection of TS/A-pc on challenge.

Our studies show that IFN-γ plays a critical role in mediating and guiding the protective immune response against the TS/A-CIITA after the initial priming of Th cells. This stems from two basic observations. First, spleen cells from TS/A-CIITA-rejecting
mice at the earliest time after rejection have a high frequency of IFN-γ-secreting tumor-specific CD4+ T cells; these cells were not present in the spleens of TS/A-pc-injected mice. Second, in GKO mice, TS/A-CIITA tumor cells are not rejected and give rise to tumors similar in frequency and growth kinetics to those generated in normal mice after injection of TS/A-pc. Moreover, TS/A-pc-injected mice present in their spleens substantial amounts of T cells producing IL-4, a paradigm cytokine of Th2 responses, which may be correlated with a suppressive immune phenotype inhibiting the generation and/or the maintaining of a protective immune response against the tumor. Taken together, these results indicate that injection of TS/A-CIITA cells is a tumor-specific Th1-oriented, IFN-γ-producer immune response is triggered, which drives and orchestrates subsequent events enabling expansion of CD8+ CTL, rejection, and immune memory responses.

The success of our approach of tumor vaccination by genetic transfer of CIITA in mammary adenocarcinoma cells opens the way to the possible use of CIITA for increasing both the inducing and the effector phases of the antitumor response.

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CIITA-Induced MHC Class II Expression in Mammary Adenocarcinoma Leads to a Th1 Polarization of the Tumor Microenvironment, Tumor Rejection, and Specific Antitumor Memory

Lorenzo Mortara, Patrizia Castellani, Raffaella Meazza, et al.


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